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[Title of the Invention] Genes involving in the restoration of fertility from cytoplasmic male sterility

[Claims]

[Claim 1] Any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 1;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3)) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

[Claim 2] Any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 2 and involves in the restoration of fertility from cytoplasmic male sterility.

[Claim 3] A DNA encoding any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[Claim 4] The DNA according to any one of Claims 1-3, wherein a cytoplasmic male sterile individual organism has cytoplasmic male sterile genes of Kosena radish and/or Ogura radish or their homologues.

[Claim 5] Any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[Claim 6] The protein according to claim 5, wherein a cytoplasmic male sterile individual organism has cytoplasmic male sterile genes of Kosena radish and/or Ogura radish or their homologues.

[Claim 7] A vector carrying a DNA according to any one of claims 1-4.

[Claim 8] A transformant having a DNA according to any one of claims 1-4 or the vector according to claim 7.

[Claim 9] The transformant according to claim 8, said transformant being a transgenic plant.

[Claim 10] A method for restoring fertility from a cytoplasmic male sterile individual organism, wherein a DNA according to any one of claims 1-4 is used.

[Claim 11] A transformant that allows controlling the expression of a male fertility restorer gene by introducing part or whole of a DNA according to any one of claims 1-4 together with an inducible promoter into a cell having both a cytoplasmic male sterile gene and a DNA according to any one of claims 1-4.

[Claim 12] A method for maintaining a cytoplasmic male sterile line using the transformant according to claim 11.

[Detailed Explanation of the Invention]

[0001]

[Industrial Field of Application]

The present invention relates to a gene involving in the restoration of fertility from cytoplasmic male sterility. More specifically, the invention relates to a gene involving in the restoration of fertility from cytoplasmic male sterile traits, which may hereinafter be referred to as cms, used for developing the first filial hybrid (hereinafter referred to as F₁), a vector containing the aforementioned gene and a transformant.

[0002]

[Prior Art]

The development of F₁ strains has been popular and widely used in practice for a wide range of main agricultural products such as cereal crops and vegetables in consideration of (1) excellent agronomic traits by hybrid vigor, (2) the uniformity of crops and (3) the protection of the benefits of breeders because acquired characteristics can be separated in the next generation.

[0003]

Methods for producing the seeds of F_1 strains include a cms – Rf seed growing system using cytoplasmic male sterility (cms) lines and male fertility restoration (which may hereinafter referred to as Rf) lines, which has been used for cereal crops such as rice, sorghum and corns and oil crops such as sunflowers using a crossbreeding or cell fusion method.

[0004]

On the other hand, an F_1 seed growing system using self incompatibility has been widely used for cruciferous vegetables. As far as rapeseed is concerned, however, an F_1 seed growing system using cms lines and Rf lines has been used because there is no stable self incompatibility.

[0005]

Much research has recently been conducted for rapeseed using cytoplasmic male sterility derived from Kosena radish (Kosena cms) and cytoplasmic male sterility derived from Ogura radish (Ogura cms). Both cms genes are encoded in genomes of mitochondria, a cytoplasmic organelle, and their base sequences are known. However, radish lags behind other plants in molecular-biological research, and few markers necessary for isolating genes are known. For this reason, it is difficult to isolate genes from a nucleus, and therefore Rf has been introduced into rapeseed using a sterility restoration line of radish by a crossbreeding or cell fusion method.

[0006]

It has been known for Rf genes that there are one or more restorer genes depending on the cms lines of plants. While the presence of both Rf1 and Rf2 are necessary for the restoration of fertility in radish, the fertility of rapeseed can be restored only with an Rf1 gene (Journal of Thremmatology 47 (additional volume 1) P186, 1997, Journal of Thremmatology 48 (additional volume 1) P197, 1998). However, the control mechanism has hardly been discovered yet.

While the base sequence of an Rf2 gene, a restorer gene for T-cytoplasm that is a cms of corns, was identified and isolated, the base sequences of Rf genes have not been known yet in the other plants.

[0007]

[Problems that the Invention is to Solve]

It has been known that the content of glucosinolate (hereinafter "GSL") becomes higher than the regulation value in F₁ strains made by a rapeseed restoration line introduced with a Rf1 gene by crossbreeding or cell fusion and the same line as a father, which is a problem in terms of practical use. It is believed that the content of GSL increases in the restoration line (Rf line) of rapeseed because a radish-derived gene involving in the biosynthesis of GSL exists in the neighborhood of the Rf1 gene, and therefore they are strongly linked genetically. GSL is contained in the pressed oil cake of rapeseed and known to cause hyperthyroid when animals are fed with it. The GSL content of rapeseed should therefore be 18 μ mole/g in North America and 20 μ mole/g in Europe on the stage of breeding.

[0008]

Furthermore, plants added with the function of herbicide resistance by gene splicing have recently been developed on a wide scale. In order to create these plants efficiently, it is insufficient to use only the rapeseed restoration line obtained by crossbreeding or cell fusion. The isolation of Rf genes, especially radish-derived Rf1 genes, has strongly been desired.

[0009]

The object of the present invention is to isolate an Rf gene, especially a radish-derived Rf1 gene, and identify its structure. Moreover, the invention is to provide a means of establishing the rapeseed restoration line using the isolated Rf gene.

[0010]

[Means of Solving the Problems]

The present inventors committed themselves to solving the aforementioned problems and succeeded in cloning an Rf1 gene from radish. Thus, they could solve the aforementioned problems.

In other words, the present invention provides any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 1;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

[0011]

The present invention also provides any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 2 and involves in the restoration of fertility from cytoplasmic male sterility.

[0012]

The present invention also provides a DNA encoding any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

In the present invention, a cytoplasmic male sterile individual organism preferably has cytoplasmic male sterile genes of Kosenia radish and/or Ogura radish or their homologues.

[0013]

The present invention also provides any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or

(2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

In the present invention, a cytoplasmic male sterile individual organism preferably has cytoplasmic male sterile genes of Kosena radish and/or Ogura radish or their homologues.

[0014]

The present invention also provides a vector containing a DNA according to the present invention.

The present invention also provides a transformant having a DNA according to the present invention or a vector according to the present invention.

The present invention also provides a method for restoring fertility from a cytoplasmic male sterile individual organism using a DNA according to the present invention.

The present invention also provides a transformant that allows controlling the expression of a male fertility restorer gene by introducing part or whole of a DNA according to the present invention together with an inducible promoter into a cell having both a cytoplasmic male sterile gene and a DNA according to the present invention.

The present invention also provides a method for maintaining a cytoplasmic male sterile line using the transformant according to present invention.

[0015]

[Mode for Implementing the Invention]

A description of a mode for implementing the present invention is given below in detail.

(1) Mode of DNA according to the invention

The DNA according to the present invention relates to any one of the following DNAs.

- (1) a DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

[0016]

Moreover, the DNA according to the present invention relates to a DNA encoding any one of the following proteins.

(1) a protein having the amino acid sequence described in SEQ ID NO: 3; or

(2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[0017]

As used herein, DNA according to the present invention may also be referred to as genes according to DNA.

The base sequence described in SEQ ID NO: 1 is a genomic DNA base sequence consisting of 8553 bases. The base sequence described in SEQ ID NO: 2 is a coding sequence assumed by SEQ ID NO: 1. The base sequence described in SEQ ID NO: 3 is an amino acid sequence encoded by the base sequence described in SEQ ID NO: 2.

[0018]

As used herein, the term "a base sequence in which one or more of bases are deleted from, added to and/or substituted for" refers to a base sequence in which 1 to 20, preferably 1 to 15, more preferably 1 to 10 and still further preferably 1 to 5 bases are deleted, added and/or substituted.

As used herein, the term "an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for" refers to an amino acid sequence in which 1 to 20, preferably 1 to 15, more preferably 1 to 10 and still further preferably 1 to 5 amino acids are deleted, added and/or substituted.

[0019]

As used herein, the term "DNA that can hybridize ...under stringent conditions" refers to the base sequence of DNA found by the colony hybridization method, plaque hybridization method or Southern blot hybridization method using DNA as a probe, which includes DNA that can be identified by performing hybridization at 65°C in the presence of 0.7 ~ 1.0 M NaCl using a filter on which colony or plaque-derived DNA or its DNA fragments are immobilized and then washing the filter at 65°C using 0.1 ~ 2 XSSC solution, wherein the composition of 1 XSSC is 150 mM NaCl and 15 mM sodium citrate.

[0020]

Hybridization can be performed by the methods described in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989; hereinafter referred to as "Molecular Cloning 2nd Ed.") and the like.

[0021]

The DNA that can hybridize under stringent conditions includes DNA that has a specified level or more of homology with the base sequence of DNA used as a probe. Here, the term "a specified level or more of homology" means not less than 70%, preferably not less than 80%, more preferably not less than 90%, more preferably not less than 93%, particularly preferably not less than 95% and most preferably not less than 97%. As used herein, the DNA having a specified level or more of homology includes both polynucleotide having the aforementioned homology and its complementary stranded polynucleotide.

[0022]

The DNA according to the present invention is DNA involving in the restoration of fertility from cytoplasmic male sterile individual organisms. More specifically, the invention provides F₁ seeds in which fertility is restored by crossbreeding a transgenic plant (Rf line) introduced with DNA according to the present invention using a gene splicing technique with an individual organism of the cytoplasmic male sterility line (cms line). The cms line preferably includes Kosena cms and Ogura cms.

[0023]

(2) Method for acquiring DNA according to the present invention

The method for acquiring DNA is not limited in the present invention. The DNA according to the invention can be isolated by a general breeding method and a general genetic engineering method well known to people skilled in the art based on information on the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 and information on the amino acid sequence described in SEQ ID NO: 3 as disclosed herein.

[0024]

Specifically, genomic DNA containing a cytoplasmic male fertility restorer gene of a suitable plant in which the gene according to the present invention can be expressed, more specifically a *Raphanus* plant including radish species or related species and more specifically a *Raphanus* plant including Kosen radish, Ogura radish, Yuanhong radish, their species and related species can be isolated from Brassica plants into which it is transfused by crossbreeding or cell fusion and more specifically from Rf rapeseed. For example, the gene according to the present invention can be isolated by first isolating DNA markers positioned in the vicinity of the Rf gene, making a genomic map showing the genetic distance between the DNA markers and the Rf gene, and then using a positional cloning method, which is also referred to as chromosome walking, of the Rf region based on the genomic map.

[0025]

This method starts with finding suitable DNA markers on a genomic DNA and then making a genomic map by measuring the distance between the Rf gene and the DNA markers. The DNA markers, which must be distinguished between paternal and maternal genomes, generally have several hundred bp in length. Also, the DNA markers need to be located on the same chromosome as the gene. It is desirable that the markers are nearly the same as the gene in terms of a mode of inheritance because of the close physical location (i.e. the markers are genetically strongly linked).

[0026]

The RFLP method was previously used as a DNA marker isolation method, but the RAPD method and the AFLP (Amplified Fragment Length Polymorphism) method, simple methods using PCR, has recently been used (Nucleic Acids Research, 1995, Vol. 23, No. 21, 4407-4414). The AFLP method is especially effective as a means of obtaining genetically strongly linked markers. Materials used for measuring the genetic distance between a gene and markers usually include an F₂ population obtained by self-fertilizing an F₁ generation crossbred between a recessive homo individual, which has no Rf1 gene, and a dominant homo individual, which has an Rf1 gene in homo and a BC₁ population obtained by crossbreeding between an F₁ generation and its parent (i.e. a recessive homo individual, which has no desired gene).

[0027]

The aforementioned recessive homo individuals include cytoplasmic male sterile *Raphanus* plants such as radish species and related species, more specifically cytoplasmic male sterile Kosena radish and Ogura radish, and Brassica plants, more specifically cms rapeseed, transfused with cytoplasmic male sterility derived from Kosena radish (Kosena cms) or cytoplasmic male sterility derived from Ogura radish (Ogura cms).

[0028]

The aforementioned dominant homo individuals include Rf line *Raphanus* plants such as radish species and related species, more specifically cytoplasmic male fertility restored Kosena radish, Ogura radish and Yuanhong radish, and Brassica plants, more specifically cms rapeseed, transfused by crossbreeding or cell fusion with a cytoplasmic male fertility restorer gene of *Raphanus* plants such as radish species and related species.

[0029]

It is desirable to analyze 100 individuals or more and more preferably 1000 individuals or more of an F₂ population obtained by self-fertilizing an F₁ generation crossbred between the aforementioned parents and a BC₁ population obtained by crossbreeding between an F₁ generation and a recessive homo individual. The more the number of individuals, the more precise a genomic map and the shorter the physical distance between DNA markers and a desired gene. This is true for an Rf gene. DNA markers of shorter physical distances can be obtained.

[0030]

Materials used for measuring genetic distances between DNA markers and an Rf gene include an F₂ population of several thousands obtained by self-fertilizing an F₁ generation of radish crossbred between cms line Kosena radish (*Raphanus sativus* cv. Kosena) and Rf line Yuanhong radish (*Raphanus sativus* cv. Yuanhong) by a method described in N. Koizuka, et al., Theor. Appl. Genet., 100:949-955, 2000. Analyzing these allows isolating DNA markers linked both sides of the Rf gene at a genetic distance of approximately 0.2 cM. As a result, it is possible to make a genomic map showing the genetic distances between markers and the Rf gene as shown in Fig. 1.

[0031]

Subsequent to making a genomic map, the corresponding genomic DNAs need to be cloned to link DNA markers on both sides of a desired gene. Since the physical distance between DNA markers and a desired gene is usually large, the region between the DNA marker and the desired gene must be covered by linking multiple clones having a genomic DNA fragment. The step of linking DNA markers with clones having a genomic DNA fragment is referred to as making a contig. This is true for a RF gene. A contig can be made by linking multiple clones having a genomic DNA fragment for DNA markers that are physically close to the Rf gene in such a way as to cover the Rf gene region.

[0032]

A group of clones having a genomic DNA fragment can be obtained by making a genomic library. Several types of vectors are usually used depending on the length of genomic DNA to be cloned to make a library using a lambda phage vector that can clone a fragment up to approximately 20 kb, a cosmid vector that can clone a relatively long fragment (~ 40 kb), BAC (Bacterial Artificial Chromosome) vector that can clone a longer fragment (100 kb or more) or the like.

[0033]

It is important for any library that a value calculated by multiplying an average length of cloned fragments by a population number of a library should be approximately four to five times more than the total length (a genome size) of a genome provided to the library. The genome size of radish is believed to be approximately 500 Mbp. In the case of a lambda phage vector, if an average length is 20 kb, a population number is in the range of $1.0 \times 10^5 \sim 1.25 \times 10^5$. In the case of a cosmid library, if an average length is 40 kb, a population number is in the range of $5.0 \times 10^4 \sim 6.25 \times 10^4$. The genome size of rapeseed is believed to be approximately 1000 Mbp. In the case of a lambda phage vector, if an average length is 20 kb, a population number is in the range of $2.0 \times 10^5 \sim 2.5 \times 10^5$. In the case of a cosmid library, if an average length is 40 kb, a population number is in the range of $1.0 \times 10^5 \sim 1.25 \times 10^5$.

[0034]

Genomic DNA to be provided to a library can be extracted from an organism containing a specific gene. In the case of a Rf gene, what can be used include Rf line *Raphanus* plants such as radish species and related species, more specifically cytoplasmic male fertility restored Kosen radish, Ogura radish and Yuanhong radish, and Brassica plants, more specifically cms rapeseed, transfused by crossbreeding or cell fusion with a cytoplasmic male fertility restorer gene of *Raphanus* plants such as radish species and related species. In general, it is most desirable to make a genomic library by extracting genomic DNA from a plant having the same Rf line as the parent used at the time of making an F₂ population and a BC₁ population. Genomic DNA can be prepared by an ordinary method such as the CTAB method (Murray, M. G. and Thompson, W. F. (1980), *Nucleic Acids Res.*, 8, 4321).

[0035]

A contig is made by isolating clones retaining a DNA marker on both sides of an Rf gene first. The clones are isolated by an ordinary method based on a genomic library. In the case of a lambda phage library, the plaque hybridization method is used. In the case of a cosmid library and a BAC library, the colony hybridization method is used. Next, using the terminal regions of an isolated clone as an indicator, a clone adjacent to the clone is isolated in order to make a contig. After making a contig, the base sequence of the contig is determined by an ordinary method.

[0036]

As genome projects have recently made great progress, so does technology to assume functional genes based on the base sequence of genomic DNA. Gene discovery programs represented by "Genscan" can assume genes with very high accuracy. Also, homology research programs represented by "Blast" can allow assuming the similarity of other genes and proteins. Thus, specific genes have been assumed and isolated using such analysis software. When it comes to an Rf gene, it is thought it possible to isolate genomic DNA and identify its base sequence using similar analysis software. Such an analysis allows clarifying a promoter on the base sequence of genomic DNA, a structural gene including intron and a terminator as well as a gene without intron (i.e. a gene to be translated into protein) and an amino acid sequence encoded by the gene. Thus, an Rf gene on a contig can be assumed with very high accuracy.

[0037]

It is possible to determine whether or not the promoter, structural gene containing intron and terminator thus obtained are the Rf gene itself based on the aforementioned genomic map, the relationship between DNA markers and the relationship between DNA markers and a contig.

[0038]

Genes to be translated into protein that can be assumed by the aforementioned method include DNA described in SEQ ID NO: 2. Based on the DNA sequence, cDNA can also be isolated by a general genetic engineering method.

[0039]

Specifically, a cDNA is prepared by an ordinary method from Brassica plants and more specifically from Rf rapeseed transfused with genomic DNA of a suitable plant in which the gene according to the present invention can be expressed, more specifically a Raphanus plant including radish species or related species and more specifically a Raphanus plant including Kosena radish, Ogura radish, Yuanhong radish, their species and related species. The cDNA corresponding to the gene of the present invention can be isolated by selecting specific clones based on the library using a proper DNA fragment unique to the gene according to the invention as a probe or an antibody of the translated product of the gene according to the invention.

[0040]

The source of cDNA includes various cells, tissues and cultured cells derived from them that express the gene according to the invention. The isolation of total RNA, the isolation and purification of mRNA and the acquisition and cloning of cDNA can be performed by an ordinary method.

A method for screening the gene according to the invention is not limited. Any ordinary method can be used.

[0041]

A probe that can be used here includes DNA chemically synthesized based on information about the base sequence of the gene according to the invention and the gene according to the invention that has already been acquired and its fragments. A sense primer and an antisense primer made based on information about the base sequence of the gene according to the invention can be used as a probe for screening.

[0042]

The nucleotide sequence of a sense primer and an antisense primer used as a probe as described above is a partial nucleotide sequence corresponding to DNA encoding the amino acid sequence expressed by SEQ ID NO: 3 and has at least continuous bases of not less than 15, preferably continuous bases of not less than 20, more preferably continuous bases of not less than 30 and most preferably continuous bases of not less than 50. Or, a positive clone having the aforementioned sequence can also be used as a probe.

[0043]

The gene according to the present invention can be obtained by combining ordinary methods used for the isolation of genes such as a DNA/RNA amplification method based on the PCR method and the RACE method represented by the 5' – RACE method.

[0044]

A primer to be used for PCR can optionally be set and synthesized by an ordinary method based on information about the sequence of the gene according to and revealed by the invention. The isolation and purification of amplified DNA/RNA fragments can follow an ordinary method as described above including gel electrophoresis.

[0045]

Furthermore, the base sequences of the gene according to the invention thus obtained or various DNA fragments can be determined by an ordinary method.

The presence of the gene according to the invention and the presence or absence of expression in individual organisms or various tissues can characteristically be detected using part or whole of the base sequence of the gene, for example.

[0046]

As described above, the gene according to the present invention includes DNA encoding the amino acid sequence expressed in SEQ ID NO: 3, but is not limited to this. Included are homologues of the gene as well.

As used herein, the term "homologues of the gene" refers to a group of related genes recognizable as one gene family based on sequence homology with the gene according to the invention (or its genetic products), the aforementioned structural characteristics and the similarity of biological functions including the allele gene of the gene.

[0047]

The gene according to the present invention is not limited to a gene having a specific base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2. It can be a base sequence formed by optionally combining codons for amino acid residues described in SEQ ID NO: 3. Codons can be selected by an ordinary method in consideration of the frequency of the use of codons by a host, for example.

[0048]

As described above, the gene according to the present invention also includes DNA that can hybridize with DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 under stringent conditions. Such DNA has a specific level or more of homology with DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2.

[0049]

The aforementioned DNA having a specific level or more of homology refers to polynucleotide and its complementary chain polynucleotide having at least 70% identity, preferably at least 90% identity, more preferably at least 95% identity and still further favorably at least 97% identity with the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 or the base sequence encoding the amino acid sequence described in SEQ ID NO: 3.

[0050]

More specifically, it is exemplified by DNA having a base sequence that hybridizes with DNA having the base sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 2 under the stringent conditions of 50°C in 0.2 XSSC containing 0.1% SDS or 60°C in 1 XSSC containing 0.1% SDS.

[0051]

Particularly, among the DNAs according to the present invention, the following DNAs can be made by any method well known to people skilled in the art including chemical synthesis, genetic engineering and mutagenesis:

- a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility;

- a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; and

- a DNA encoding a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility. For example, mutated genes can be obtained by carrying out the mutagenesis of DNAs having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2.

[0052]

Mutated genes can be produced by any well-known method such as a method using random mutants, mutants having a target or synthesized genes (New Genetic Engineering handbook, Experimental Medicine, Additional Volume, Yodosha Co., Ltd.).

Included are a method of contacting DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 with a chemical (a mutagen), a method of irradiating ultraviolet and genetically engineering methods. The site-specific mutagenesis, one of genetically engineering methods, is very useful because it is a method of introducing a specific mutation into a specific position and can be carried out in accordance with the method described in Molecular Cloning 2nd Ed.

[0053]

(3) A vector containing the DNA according to the present invention

The DNA according to the present invention can be used as a recombinant vector by recombining it into a proper vector. The A vector can be either an expression vector or a non-expression vector and can be selected depending on the purpose.

A cloning vector is preferably one that can autonomously replicate in the Escherichia coli k12 strain including a phage vector and a plasmid vector. An expression vector for Escherichia coli can also be used as a cloning vector. Specifically, the following are included: ZAP Express [manufactured by Stratagene Corporation, Strategies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], Lambda ZAP 11 (manufactured by Stratagene Corporation), λ gt10, λ gt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)], λ TriplEx (manufactured by Clone Tech Corporation), λ Excell (manufactured by Pharmacia Corporation), pT7T318U (manufactured by Pharmacia Corporation), pcD2 [Mol. Gen. Biol., 3, 280 (1983)], pMW218 (manufactured by Wako Pure Chemical Industries, Ltd.), pUC118 (manufactured by Takara Shuzo Co., Ltd.), pEG400 [J. Bac, 172, 2392 (1990)] and pQE-30 (manufactured by Qiagen Corp.).

[0054]

An expression vector can be selected in consideration of the combination with a host and is preferably one that can autonomously replicate in the host cell or can be recombined into a chromosome and contains a promoter in the position that allows transcribing the gene according to the present invention.

In the case that a bacterium is used as a host cell, an expression vector that allows expressing DNA is preferably a recombinant vector that can autonomously replicate in the bacterium and is composed of a promoter, a ribosome binding sequence, the aforementioned DNA and a transcription termination sequence. A promoter controlling gene may be contained.

[0055]

Expression vectors for bacteria include pBTrP2, pBTac1, and pBTac2 (these three are available from Boehringer Mannheim Corporation), pKK233-2 (manufactured by Pharmacia Corporation), pSE280 (manufactured by Invitrogen Corporation), pGEMEX-1 (manufactured by Promega Corporation), pQE-8 (manufactured by Qiagen Corp.), pQE-30 (manufactured by Qiagen Corp.), pKYP10 (Japanese Unexamined Patent Publication No. S58-110600), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescriptII SK+, pBluescriptII SK(-) (manufactured by Stratagene Corporation), pTrs30 (FERMBP-5407), pTrs32 (FERM BP-5408), pGEX (manufactured by Pharmacia Corporation), pET-3 (manufactured by Novagen Corporation), pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTp5, pC194, pUC18 [Gene, 33, 103 (1985)], pUC19 [Gene, 33, 103 (1985)], pSTV28 (manufactured by Takara Shuzo Co., Ltd.), pSTV29 (manufactured by Takara Shuzo Co., Ltd.), pUC118 (manufactured by Takara Shuzo Co., Ltd.), pPA1 (Japanese Unexamined Patent Publication No. S63-233798), pEG400 [J. Bacteriol., 172, 2392 (1990)] and pQE-30 (manufactured by Qiagen Corp.). Promoters for bacteria include promoters originated from Escherichia coli and phages such as a trp promoter (P trp), a lac promoter (P lac), a P_L promoter, a P_R promoter and P_{SE} promoter, a SP01 promoter, a SP02 promoter and a penP promoter.

[0056]

Expression vectors for yeasts include Yep13 (ATCC37115), Yep24 (ATCC37051), Ycp50 (ATCC37419), pHS19 and pHS15. Promoters for yeasts include a PH05 promoter, a PGK promoter, a GAP promoter, an ADH promoter, a gal1 promoter, a gal10 promoter, a heat shock protein promoter, an MFa1 promoter and a CUP1 promoter.

[0057]

Expression vectors for animal cells include pcDNA1, pcDM8 (available from Funakoshi Corporation), pAGE107 (Japanese Unexamined Patent Publication No. H3-22979; Cytotechnology, 3, 133, (1990)), pAS3-3 (Japanese Unexamined Patent Publication No. H2-227075), pCDM8 [Nature, 329, 840 (1987)], pc DNAI/AmP (manufactured by Invitrogen Corporation), pREP4 (manufactured by Invitrogen Corporation), pAGE103 [J. Biochem., 101, 1307 (1987)] and pAGE210. Promoters for animal cells include a promoter for an IE (immediate early) gene of cytomegalovirus (human CMV), a promoter for retrovirus, a metallothionein promoter, a heat shock promoter and a SRa promoter.

[0058]

Expression vectors for plant cells include pIG121-Hm [Plant Cell Report, 15, 809-814 (1995)] and pBI121 [EMBO J. 6, 3901-3907 (1987)]. Promoters for plant cells include a cauliflower mosaic virus 35S promoter [Mol. Gen. Genet (1990) 220, 389-392]. The transformation of plants will be described in detail below.

[0059]

(4) A transformant having the DNA according to the present invention

A transformant having the DNA according to the present invention can be made by introducing the aforementioned vector and preferably an expression vector into a host.

Host bacterial cells include genus *Escherichia*, genus *Corynebacterium*, genus *Brevibacterium*, genus *Bacillus*, genus *Microbacterium*, genus *Serratia*, genus *Pseudomonas*, genus *Agrobacterium*, genus *Alicyclobacillus*, genus *Anabaena*, genus *Anacystis*, genus *Arthrobacter*, genus *Azobacter*, genus *Chromatium*, genus *Erwinia*, genus *Methylobacterium*, genus *Phormidium*, genus *Rhodobacter*, genus *Rhodopseudomonas*, genus *Rhodospirillum*, genus *Scenedesmun*, genus *Streptomyces*, genus *Synnecoccus* and genus *Zymomonas*. The methods for introducing a recombinant vector into a bacterial host include a method using a calcium ion and a protoplast method.

[0060]

Host yeast cells include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*.

Any method for introducing a recombinant vector into a host yeast cell can be used including an electroporation method, a spheroplast method and a lithium acetate method.

[0061]

Host animal cells include *Namalwa* cells, COS1 cells, COS7 cells and CHO cells.

Any method for introducing a recombinant vector into an animal cell can be used including an electroporation method, calcium phosphate method and a lipofection method. Transformants using plant cells will be described below.

[0062]

(5) Production of protein according to the present invention

The present invention relates to any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[0063]

The protein according to the present invention can be acquired by culturing a transformant containing the gene according to the present invention, allowing producing and accumulating the protein according to the present invention in the culture, and then harvesting the protein from the culture.

[0064]

A transformant containing the gene according to the present invention can be cultured by any ordinary method used for culturing a host.

If the transformant according to the present invention is prokaryote such as *Escherichia coli* or eukaryote such as yeast, a medium used for culturing these microorganisms may be a natural or synthetic medium as far as the medium contains carbon sources, nitrogen sources, inorganic salts and the like that can be utilized by the microorganisms and allows efficiently culturing a transformant. The culture condition is preferably aerobic such as shake culture and submerged culture. The culture temperature is usually in the range of 15 ~ 40°C. The culture time is usually 16 hours to 7 days. During the culture, pH should be maintained 3.0 ~ 9.0, which can be adjusted using inorganic or organic acids, alkali solution, urea, calcium carbonate, ammonia or the like. As the need arises, a medium can be added with antibiotics such as ampicillin and tetracycline.

[0065]

A transformant acquired by using an animal cell as a host cell can be cultured in any widely used medium such as a RPM 11640 medium [The Journal of the American Medical Association, 199, 519 (1967)], an Eagle's MEM medium [Science, 122, 501 (1952)], a DMEM medium [Virulorogy, 8, 396 (1959)], a 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] and media made by adding fetal bovine serum to these media. The culture is usually performed at pH 6 ~ 7 at 30 ~ 40 °C for 1 to 7 days in the presence of 5% CO₂. As the need arises, a medium can be added with antibiotics such as kanamycin and penicillin.

[0066]

A transformant acquired by using a plant cell as a host cell can be cultured in a MS medium, a R2P medium or any other media usually used depending on the type of plant species. The culture is usually performed at pH 6 ~ 8 at 15 ~ 35°C for 1 to 21 days. As the need arises, a medium can be added with antibiotics such as kanamycin and hygromycin.

[0067]

The protein according to the present invention involving in the restoration of fertility from cytoplasmic male sterile individual organisms can be isolated from the culture containing a transformant and then purified using ordinary isolation and purification methods for protein.

For example, if the protein according to the present invention is expressed in cells in the state of dissolution, the cells are collected by centrifugal separation after the completion of the culture, suspended in an aqueous buffer solution, and then smashed with a French press, a Manton Gaulin homogenizer or a Dyno-mill to obtain a cell free extract. Purified samples can be produced from the supernatant obtained by performing the centrifugal separation of the cell free extract using an ordinary protein isolation and purification method such as a solvent extraction method, a salt precipitation method using ammonium sulfate or the like, a desalination method, a precipitation method by an organic solvent, anion exchange chromatography using resin including diethylaminoethyl (DEAE) Sepharose and DIAION HPA-75 (Mitsubishi Kasei Corporation), cation exchange chromatography using resin including S-Sepharose FF (Pharmacia Corporation), hydrophobic chromatography using resin including Butyl Sepharose and Phenyl Sepharose, a gel filtration method using molecular sieves, affinity chromatography, a chromatofocusing method or electrophoresis including isoelectric electrophoresis or a combination thereof.

[0068]

If protein is expressed in cells in the form of insoluble substance, the cells are collected, smashed and then centrifuged. The protein is collected from the precipitated fraction. Then, the insoluble protein is solubilized by a protein denaturizing agent. The solubilized solution is dialyzed or diluted into its normal stereostructure with a solution containing no protein denaturizing agent or a solution in which the concentration of a protein denaturizing agent is so diluted that it cannot denature protein to change the protein. Subsequently, purified samples can be produced by the same isolation and purification method as described above.

[0069]

If the protein according to the present invention or its derivative such as sugar modification is secreted extracellularly, the protein or its derivative such as a glycosylated derivative can be collected from the supernatant of the culture. In other words, the culture is processed by the centrifugal separation method and the like, as described above, to obtain a soluble fraction, and then purified samples can be produced from the soluble fraction by the same isolation and purification method as described above.

[0070]

Furthermore, the protein according to the present invention can be produced by chemical synthesis such as an Fmoc method (a Fluorenyl methyloxy carbonyl method) and a tBoc method (a t-butyloxy carbonyl method). Also, the protein can be synthesized using peptide synthesizers such as those obtainable from Sowa Trading Co., Inc. (manufactured by Advanced Chem Tech Corporation in USA), Perkin-Elmer Japan (manufactured by Perkin-Elmer Corporation in USA), Pharmacia Biotech (manufactured by Pharmacia Biotech Corporation in Sweden), Aloka (manufactured by Protein Technology Instrument Corporation in USA), Kurabo Industries Ltd. (manufactured by Synthecell-Vega Corporation), Nihon PerSeptive Ltd. (PerSeptive Corporation in USA) and Shimadzu Corporation.

[0071]

(6) Plant transformants containing the DNA according to the present invention

The base sequence described in SEQ ID NO: 1 is the original plant genomic base sequence. This base sequence operably contains a promoter and a terminator necessary for the expression of a gene. In the case of the direct introductory method, the gene can be cloned into a general cloning vector such as cosmid pWE15 (manufactured by Strategen Corporation). In the case of using Agrobacterium, the gene can be cloned into a general vector for plant transformation such as pBI121 (manufactured by Clontec Corporation).

[0072]

Also, plant cells can be introduced with DNA having a base sequence formed by removing some introns from the aforementioned base sequence, DNA having a base sequence formed by removing almost all introns from the aforementioned base sequence, DNA described in SEQ ID NO: 2 or DNA encoding protein described in SEQ ID NO: 3.

[0073]

Moreover, the promoter and terminator may be substituted by a promoter and a terminator that function in the well-known plant cells.

In the case that DNA described in SEQ ID NO: 2 or DNA encoding the protein described in SEQ ID NO: 3 is introduced into plant cells, a promoter and a terminator are required in addition to the DNA. A general expression vector used very often includes pBI121 (manufactured by Clontec Corporation). This vector uses a 35S promoter of cauliflower mosaic virus as a promoter and a Nopaline synthase terminator in the Ti plasmid of A. tumefaciens as a terminator. A promoter necessary for the expression may be an rbcs promoter that widely exists in plants besides the aforementioned 35S promoter of cauliflower mosaic virus, more preferably a promoter that expresses in the growing period of pollens and still further preferably the original promoter existing in the upstream of the gene. A promoter may be a 35S terminator of cauliflower mosaic virus besides the aforementioned Nopaline synthase terminator and more preferably the original terminator existing in the downstream of the gene.

[0074]

In the following Examples, the present inventors made vectors for plant transformation in order to introduce into a plant the original form of DNA for an Rf gene containing introns between the original promoter and the original terminator in the genome as described in SEQ ID NO: 1. The base sequence described in SEQ ID NO: 1 was cut out of a To3-2 clone, part of the contig, with a restriction enzyme and subcloned to a proper cloning vector. The subcloned fragment was introduced into pKM424, a vector for plant transformation, to obtain a vector that allows introducing the fragment into a plant. This vector was introduced into *Agrobacterium* for plant transformation. Thus, the DNA fragment could be introduced into a plant genome by infecting a plant with the *Agrobacterium* containing this vector.

[0075]

Plants used for the gene according to the present invention include oil crops such as rapeseed, sunflower, soybean and palm, cereals such as rice, corn and wheat, flowering plants such as tobacco and petunia and various vegetables such as tomato, broccoli, cabbage, Chinese cabbage and carrot,

Among those plants, tomato and genus *Brassica* including rapeseed, cabbage, Chinese cabbage and broccoli are preferred. Particularly, rapeseed, cabbage, Chinese cabbage and broccoli are preferred. The most preferred plant is rapeseed.

[0076]

In the present invention, the transgenic plant source includes seeds, sprouts, seedlings, calluses, cultured cells and plant bodies. For example, plant parts to be selected are as follows: sprouts or protoplast in rapeseed; sprouts, calluses or cultured cells in soybean; sprouts in sunflower; calluses or cultured cells in palm; sprouts, calluses, cultured cells or protoplast in rice; sprouts, seedlings, calluses, cultured cells or protoplast in corn; sprouts, calluses or cultured cells in wheat; sprouts, calluses, cultured cells or protoplast in cabbage and broccoli; and sprouts, calluses, cultured cells or protoplast in carrot. Thus, plant parts may optionally be selected depending on the type of plants as usually performed by people skilled in the art.

[0077]

Plants may be transformed by any ordinary method. For example, a vector can be introduced into a plant by first introducing the vector into *Agrobacterium* and then infecting a plant with the *Agrobacterium*. Or, a vector can directly be introduced into cells using an electroporation method, a DEAE dextran method, a calcium phosphate method, a polyethylene glycol method or a particle gun method.

[0078]

A description of a gene introduction method preferred for rapeseed is given below.

The hypocotyls of rapeseed germinated sterilely in the MS medium containing sugar such as sucrose as a carbon source is precultured in the medium containing 2,4 – dichlorophenoxyacetic acid and sucrose. Agrobacterium proliferated in the YEB medium is collected by centrifugation and resuspended in the MS medium containing sucrose. The suspension is added with the hypocotyls of rapeseed to be shaken. The hypocotyls thus obtained are brought back to the preculture medium to cocultivate for three days. Subsequently, they are transferred to a selective medium containing plant hormones such as zeatin and benzylaminoprine for selection. The green regeneration buds thus obtained are cultivated in an elongation medium optionally containing plant hormones such as benzylaminoprine followed by a rooting medium optionally containing naphthaleneacetic acid and plant hormones such as benzylaminoprine to obtain regenerated individual organisms. The crossbreeding of these individual organisms with individual organisms of the cms line provides a fertility-restored F1 hybrid.

[0079]

Thus, the introduction of the DNA according to the present invention into plants allows restoring fertility from cytoplasmic male sterile individual organisms.

The confirmation of expression is possible by crossbreeding the aforementioned regenerated individual organisms with rapeseed of the cms line and then examining the fertility of its offspring. It is, however, preferable to perform transformation by using rapeseed containing the cms cytoplasm as raw material in terms of time and operation, because fertility can be examined after transferring the aforementioned transformants (regenerated individual organisms) that have formed roots to soil containing ordinary fertilizer to make them bloom.

If the aforementioned transformation is performed using cells or tissues and preferably hypocotyls, seminal leaves, leaves, pollens, cultured cells, calluses or protoplasts of rapeseed containing the cms cytoplasm, sterility-restored plants can be obtained by transferring the plants (regenerated individual organisms) acquired above to soil containing ordinary fertilizer to make them bloom.

That is, plants having a nucleus introduced with DNA according to the present invention can be obtained using cms cells by introducing the DNA to the cms cells by the aforementioned gene introduction method, selecting cells having a nucleus introduced with the DNA using a marker such as an antibiotic (e.g. kanamycin) resistant or herbicide resistant selective marker, and then cultivating them in an elongation medium or a rooting medium as described above. Such plants are restored in fertility.

Furthermore, the expression of DNA according to the present invention can be controlled specifically and temporarily by introducing part or whole of the gene according to the present invention together with an inducible promoter into cells containing cms cytoplasm and DNA according to the present invention. Thus, a new hybrid seed production system can be established that does not require the male sterility maintaining line (maintainer) necessary for the production of hybrid seeds.

Since rapeseed of the cms line is sterile, a maintainer in which neither cms nor Rf is involved is required for proliferating and maintaining the cms line. In other words, plants of three lines (i.e. an Rf line, a cms line and a maintainer) are conventionally required for the production of hybrid seeds. However, since an Rf gene was isolated and identified by the present invention, it has become possible to construct the cms line that can be proliferated and maintained with no maintainer by inducing a promoter with a chemical at the time of the production of hybrids in order to control the expression of the restored gene.

Specifically, part or full length of the gene according to the present invention is introduced into an externally inducible promoter (e.g. a promoter sensitive to a chemical) in antisense or sense direction, and then cells containing the cms cytoplasm and DNA according to the present invention are transformed using the above-said vector.

Cells containing the cms cytoplasm and DNA according to the present invention can be produced not only by transforming cells containing the cytoplasm with DNA according to the present invention as described above, but also by crossbreeding the cms line and the Rf line.

The aforementioned inducible promoter is known in Japanese Unexamined Patent Publication H6-46697, for example. Also, the aforementioned methods may be used for the preparation of a vector and transformation.

The aforementioned transformant containing the cms cytoplasm and DNA according to the present invention and introduced with part or whole of DNA according to the present invention together with an inducible promoter is fertile by virtue of an Rf gene because the promoter has not been induced yet. This line can be maintained by self-fertilization. However, this plant can be used as the cms line at the time of the production of hybrid seeds by applying a chemical that has the ability of inducing the promoter because the expression of the Rf gene is inhibited by the induction of the promoter, whereby the plant become male sterile.

Accordingly, the proliferation and maintenance of the cms line has become possible by self-fertilization using the present method. Of three lines conventionally required for the production of hybrid seeds, the maintainer has become unnecessary, resulting in a significant decrease in the production cost.

A description of examples is given below in further detail. However, the present invention is not limited to those examples.

[0080]

[Examples]

Example 1: Isolation of DNA markers linked to the cytoplasmic male fertility restorer gene and preparation of a genomic map

In order to isolate a fertility restorer gene (an Rf gene), DNA markers in the neighborhood of the Rf gene needs to be isolated and then a genomic map showing the genetic distance between the DNA markers and the Rf gene need to be prepared. To start with, the positional cloning of the Rf region was performed.

[0081]

In order to isolate DNA markers, we performed the AFLP (Amplified Fragment Length Polymorphism) method (Nucleic Acids Research, 1995, Vol. 23, No. 21 4407-4414) and used an AFLP Analysis System I AFLP Starter Primer Kit that complies with the AFLP method. As for the material used for measuring the genetic distance of the markers, we used approximately 2100 individual organisms of an F2 population obtained by self-fertilizing 8 individual organisms of the F1 generation of radish obtained by crossbreeding one individual organism of Kosena radish (*Raphanus sativus* cv. Rosena) of the cms line and one individual organism of Yuanhong radish (*Raphanus sativus* cv. Yuanhong) of the Rf line in accordance with the method described in N. Koizuka, et al. Theor Appl Genet, 100:949 – 955, 2000. As a result, we could isolate five markers on both sides of the Rf gene with a genetic distance of approximately 0.2 cM. Fig. 1 shows the genetic distance between each DNA marker and the Rf gene.

[0082]

Example 2: Preparation of a contig based on the genomic map and an analysis of the Rf gene

Subsequent to the preparation of the genomic map, the genomic DNA corresponding to the position needs to be cloned and DNA markers on both sides of the Rf gene linked. We prepared a contig covering the area between the DNA markers and the Rf gene by linking multiple clones having a genomic DNA fragment since the distance between the DAN markers and the Rf gene was large.

[0083]

A group of clones having a genomic DNA fragment is called a genomic library. We made two types of libraries. We prepared genomic DNA by the CTAB method (Murray, M. G. and Thompson, W. F. (1980) *Nucleic Acids Res.*, 8, 4321) from Yuanhong radish, as a DNA donor, that is the same as the parent used at the time of the preparation of the F2 population. We made a lambda phage library of 20 kb in average length and 1.5×10^5 in population number using a λ DASHII vector (manufactured by Strategene corporation) as a lambda vector. We also prepared a cosmid library of 40 kb in average length and 5.5×10^4 in population number using a pWEB::TNC vector (manufactured by Epicentre Technologies Corporation) as a cosmid vector.

[0084]

In order to make a contig, we first isolated a lambda clone from the aforementioned lambda phage library by the plaque hybridization method using DNA markers on both sides of the Rf gene. We also isolated a cosmid clone from the cosmid library by the colony hybridization method in order to complete a contig that covers DNA markers on both sides as shown in Fig. 1. Base sequences were determined for cosmid clones NIT7/2 and T03-2, part of the contig, by an ordinary method.

[0085]

Next, we analyzed the base sequences of the cosmid clones NIT7/2 and T03-2, part of the cosmid, in consideration of a parameter for Arabidopsis, which is similar to radish in the DNA sequence and whose genomic sequence was recently determined, using "Genscan" (manufactured by Mitsubishi Space Software Corporation). As a result, we found a promoter portion that was thought to express the Rf gene, a structural gene portion containing introns and a terminator portion. Furthermore, we obtained a gene without introns to be translated into protein and the amino acid sequence of the gene. The promoter portion, structural gene portion containing introns and terminator portion are believed to be the Rf gene itself in consideration of their locations based on the aforementioned genomic map, relationship between DNA markers and the relationship between DNA markers and the contig.

[0086]

Example 3: Subcloning of the Genomic DNA area

We then separated a HpaI-SwaI fragment (8553 bp) of the 1st base ~ the 8553rd base described in SEQ ID NO: 1 that fully contains the portion from promoter to terminator assumed by "Genscan" using gel electrophoresis with agarose (manufactured by FMC Corporation) used for fragment recovery. We recovered DNA after digesting gel containing the DNA fragment with a gel catabolic enzyme (manufactured by Epicentre Technologies Corporation). We added dA to the 3' terminus using Taq DNA polymerase (manufactured by Takara Shuzo Co., Ltd.) and subcloned to a pGEM-T easy vector (manufactured by Promega Corporation) to obtain cds6/pGEM-Teasy. The following explains this procedure in detail.

[0087]

We added 1 µg of NIT7/2 cosmid DNA and 10 unit of a restriction enzyme HpaI (manufactured by Takara Shuzo Co., Ltd.) to 100 µl of 1 x K restriction enzyme buffer solution (20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM Dithiothreitol and 100 mM of KCl) and heated the solution at 37°C for one hour.

[0088]

After heating, we added 10 µl of 3 M sodium acetate (pH 5.6) and 250 µl of ethanol and stirred the solution. After cooling the solution at -80°C for 5 minutes, we centrifuged at 15000 rpm at 4°C for 15 minutes. We then removed the supernatant, slowly added 1 ml of 70% ethanol, and centrifuged at 15000 rpm at 4°C for 15 minutes. After removing the supernatant, we dried the precipitate for 5 minutes using a spin vacuum drier and then added 89 µl of sterile water to the recovered DNA precipitate in order to dissolve it.

[0089]

To the dissolved DNA solution, we added 10 µl of 10 X H restriction enzyme buffer solution (500 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 10 mM Dithiothreitol and 1000 mM NaCl) and 1 µl of 10 unit/µl restriction enzyme SmaI (manufactured by Takara Shuzo Co., Ltd.) and heated the solution at 25°C for one hour. We then added 11 µl of 10 X loading buffer solution (1% SDD, 50% Glycerol and 0.05% Bromophenol Blue).

[0090]

We mixed 1.2 g of SeaPlaqueGTG agarose (manufactured by FMC Corporation), a low melting point agarose, and 150 ml of 1 X TAE buffer solution (40 mM Tris-acetate and 1 mM EDTA), heated at 100°C to dissolve the agarose, and then cooled to 45°C while stirring. We set combs of 300 mm wide and 1 mm thick in a gel tray of 14 x 15 cm, poured the cooled gel and solidified it. We then poured the DNA added with loading dye into the gel combs and performed electrophoresis at 1 X TAE and 30 V/30 cm for 18 hours.

[0091]

After the electrophoresis, the gel was transferred to 0.5 µg/ml of ethidium bromide / 1 x TAE solution and dyed for 30 minutes. We put the gel on a transilluminator irradiated with long wave UV of 365 nm and cut out a specific fragment of 4126 bp with a sterile scalpel. We then cut the gel into fragments of approximately 1 by 1 millimeters square, transferred them to a 2 ml microtube weighed in advance and weighed the gel.

[0092]

We added 1 µl of 50 X GELase buffer (2M Bis-Tris (pH 6.0) and 2M NaCl) relative to 50 mg of the gel. We then put a tube containing the gel in a dry heat block heated at 68°C and heated it for 10 minutes while putting the tube upside down from time to time and stirring to completely dissolve the gel. We transferred the tube to a dry heat block heated at 45°C and heated for 5 minutes while putting the tube upside down from time and stirring. To this tube, we added 1 unit of GELase (manufactured by Epicentre Technologies Corporation) relative to 200 mg of the gel and heated for 30 minutes in the dry heat block heated at 45°C while putting the tube upside down from time and stirring.

[0093]

We added 10 M ammonium acetate (pH 7.0), which was 1/3 by volume of the gel, stirred and centrifuged at 15000 rpm for 5 minutes. We then transferred the supernatant to a new 2 ml microtube and added ethanol, which was twice by volume of the supernatant. After stirring the tube, we centrifuged at 15000 rpm at 4°C for 20 minutes. We removed the supernatant, added 1 ml of 70% ethanol slowly and centrifuged at 15000 rpm at 4°C for 5 minutes. We then removed the supernatant and dried the precipitate for 5 minutes using a spin vacuum dryer. Subsequently, we added 20 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) to dissolve it completely.

[0094]

To 20 µl of the recovered DNA solution, we added 55 µl of sterile water, 10 µl of 10 X PCR buffer solution (100 mM Tris-HCl (pH 8.3) and 500 mM KCl), 6 µl of 25 mM MgCl₂, 8 µl of 2.5 mM dNTP mix, 1 µl of 5 unit/µl rTaq DNA polymerase (manufactured by Takara Shuzo Co., Ltd., mixed them, and heated at 72°C for 30 minutes to add dATP to the 3' terminus.

[0095]

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100 µl of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit.

[0096]

We mixed 5 µl of the purified cloning fragment, 1 µl of 50 ng/µl pGEM-T easy vector (manufactured by Promega Corporation) and 6 µl of DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) I solution and incubated at 16°C for 30 minutes.

[0097]

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) together with 100 µl of sterile water and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100 µl of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit.

[0098]

We allowed the recovered DNA in the tube to stand on the ice for cooling, put 30 µl of *Escherichia coli* for electroporation (manufactured by Gibco BRL corporation) in the tube and mixed softly. We transferred the *Escherichia coli* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.25 kv, 129 Ω and 50 µF using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500 µl of SOC medium (manufactured by Gibco BRL) warmed at 37°C to the cuvette. We transferred the *Escherichia coli* to a 10 ml culture tube and cultured at 37°C for one hour by shaking. We then spread the cultured *Escherichia coli* on an LB agar medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 100 µg/ml of Ampicillin (manufactured by Wako Pure Chemical Industries, Ltd.), 20 µg/ml of X-Gal (Takara Shuzo Co., Ltd.) and 1 mM IPTG (Takara Shuzo Co., Ltd.) and cultured at 37°C for 18 hours or more.

[0099]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with 100 µg/ml of Ampicillin at 37°C for 18 hours or more. We extracted plasmid DNA from the cultured *Escherichia coli* by an ordinary method. We then confirmed that the specific fragment was cloned to the plasmid DNA by cutting it with a restriction enzyme EcoRI (manufactured by Takara Shuzo Co., Ltd.) and referred to it as *cds6/pGEM-T easy*.

[0100]

We cultured the *Escherichia coli* DH10B containing *cds6/pGEM-T easy* in 100 ml of LB medium added with 100 µg/ml of Ampicillin at 37°C for 18 hours and then purified by the Alkali SDS method using a Quiagen Midi kit (manufactured by Quiagen Corporation).

[0101]

Example 4: Preparation of a vector for plant transformation

After cutting *cds6/pGEM-T easy* with a restriction enzyme EcoRI, we separated the DNA from the vector by gel electrophoresis using agarose for fragment recovery, cloned the recovered DNA to the EcoRI site of a vector pKM4224 for plant transformation and referred to the vector for plant transformation *cds6/pKM424*. The following will explain it in detail.

[0102]

We added 1µg of cds6/pGEM-T easy DNA and 10 unit of a restriction enzyme EcoRI (manufactured by Takara Shuzo Co., Ltd.) to 100 µl of 1 x H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM Dithithreitol and 100 mM of NaCl) and heated the solution at 37°C for one hour.

We isolated and recovered an EcoRI fragment containing cds6 from cds6/pGEM-T easy in the same way as described in recovering the aforementioned HpaI-SwaI.

[0103]

We added 1µg of pKM424, a vector for plant transformation, and 10 unit of a restriction enzyme EcoRI (manufactured by Takara Shuzo Co., Ltd.) to 100 µl of 1 x H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM Dithithreitol and 100 mM of NaCl) and heated the solution at 37°C for one hour. After heating, we added 1 µl of 1 M Tris-HCl (pH 8.0) and 1 unit of Bacterial Alkaline Phosphatase (manufactured by Takara Shuzo Co., Ltd.), mixed them and heated at 50°C for one hour for dephosphorylation.

[0104]

We then added phenol/chloroform saturated with 200 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and stirred violently. After centrifuging at 15000 rpm for 5 minutes, we transferred the supernatant to a new tube. We repeated the same process to remove protein. We added 20 µl of 3 M sodium acetate (pH 5.6) and 500 µl of ethanol and stirred the solution. After cooling the solution at -80°C for 5 minutes, we centrifuged at 15000 rpm at 4°C for 15 minutes. We then removed the supernatant, slowly added 1 ml of 70% ethanol, and centrifuged at 15000 rpm at 4°C for 15 minutes. After removing the supernatant, we dried the precipitate for 5 minutes using a spin vacuum drier. We then dissolved the precipitate completely by adding 100 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) to make a concentration of 10 ng/µl.

[0105]

We mixed 10 µl of the purified EcoRI fragment, 1 µl of the dephosphorylated pKM424 vector and 11 µl of DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) I solution and incubated at 16°C for 30 minutes.

[0106]

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) together with 100 µl of sterile water and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100 µl of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit.

[0107]

We allowed the recovered DNA in the tube to stand on the ice for cooling, put 30 μ l of *Escherichia coli* for electroporation (manufactured by Gibco BRL corporation) in the tube and mixed softly. We transferred the *Escherichia coli* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.25 kv, 129 Ω and 50 μ F using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500 μ l of SOC medium (manufactured by Gibco BRL) warmed at 37°C to the cuvette. We transferred the *Escherichia coli* to a 10 ml culture tube and cultured at 37°C for one hour by shaking. We then spread the cultured *Escherichia coli* on an LB agar medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 50 μ g/ml of Spectinomycin (manufactured by Sigma Corporation) and cultured at 37°C for 18 hours or more.

[0108]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with 50 μ g/ml of Spectinomycin at 37°C for 18 hours or more. We extracted plasmid DNA from the cultured *Escherichia coli* by an ordinary method. We then confirmed that the specific fragment was cloned to the plasmid DNA by cutting it with a restriction enzyme BamHI (manufactured by Takara Shuzo Co., Ltd.) and referred to it as cds6/pKM424.

We cultured the *Escherichia coli* DH10B containing cds6/ pKM424 in 250 ml of LB medium added with 50 μ g/ml of Spectinomycin at 37°C for 18 hours and then purified by the Alkali SDS method using a Qiagen Midi kit (manufactured by Qiagen Corporation).

[0109]

Example 5: Introduction of a vector for plant transformation into *Agrobacterium*

We prepared competent cells of *Agrobacterium* and introduced the aforementioned cds6/pKM424 vector into the *Agrobacterium* EHA101 for plant transformation thus prepared.

[0110]

We prepared the competent cells for electroporation of *Agrobacterium* EHA101 by the method as shown below. Westreaked *Agrobacterium* EHA101 on the LB agar medium and cultured at 28°C for 24 hours or more to obtain single colonies. We put colonies of approximately 1 mm in diameter in a 50 ml centrifuge tube containing 20 ml of the LB medium and cultured at 28°C for 40 hours by shaking. After 40 hours, we opened and then shut the lid of the centrifuge tube and cultured for 4 more hours in the same way. To the tube disposed of the supernatant, we put 40 ml of iced 10% sterile glycerol to resuspend the bacterial cells, centrifuged at 1500 x g at 4°C and harvested the cells. We repeated the same process twice. To the bacterial cells thus obtained, we added 500 μ l of iced 10% sterile glycerol to resuspend the cells. Then, we poured 100 μ l each of the cells into sterile microtubes. After freezing them with liquid nitrogen, the cells were preserved in a freezer at -80°C.

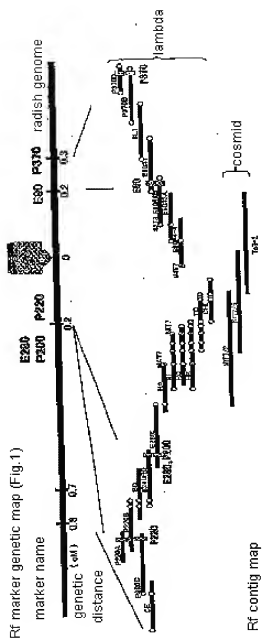
[0111]

We melted the competent cells for electroporation of *Agrobacterium* EHA101 on the ice, put 40 μ l of the electrocompetent cells in a 1.5 ml tube iced in advance, added 100 ng of the plasmid DNA of *cds6/pKM424* and mixed them mildly. We transferred the *Agrobacterium* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.44 kv, 129 Ω and 50 μ F using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500 μ l of SOC medium (manufactured by Gibco BRL) warmed at 30°C to the cuvette. We transferred the *Agrobacterium* to a 10 ml culture tube and cultured at 37°C for one hour by shaking. We then spread the cultured *Agrobacterium* on an 2 X LB agar medium (2% Bacto-Tryptone, 1% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 50 μ g/ml of Kanamycin (manufactured by Wako Pure Chemical Industries, Ltd.), 25 μ g/ml of Chloramphenicol (manufactured by Wako Pure Chemical Industries, Ltd.), 50 μ g/ml of Spectinomycin (manufactured by Sigma Corporation) and 2.5 μ g/ml of Tetracycline (manufactured by Sigma Corporation) and cultured at 30°C for 24 hours or more.

[0112]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with 50 μ g/ml of Kanamycin, 25 μ g/ml of Chloramphenicol, 50 μ g/ml of Spectinomycin and 2.5 μ g/ml of Tetracycline at 30°C for 24 hours or more. We extracted plasmid DNA from the cultured *Agrobacterium* by an ordinary method. We then confirmed that the *cds6/pKM424* was introduced into *Agrobacterium* by cutting it with a restriction enzyme BamHI (manufactured by Takara Shuzo Co., Ltd.). The confirmed clones were added with the equal amount of 80% sterile glycerol in the solution cultured for 24 hours, preserved at -80°C and used for the transformation of rapeseed.

[0113]



[Document Title] Abstract

[Abstract]

[Problems] To isolate an Rf gene, especially an Rf1 gene, originated from radish and identify its structure.

[Means of Solving the Problems] Any one of the following DNAs:

(1) a DNA having the base sequence described in SEQ ID NO: 1;

(2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or

(3)) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

[Selected Drawing] None

Applicant's Personal History

Identification No. [000005968]

1. Date of Change: October 20, 1994

[Reason of the Change] Change of name

Address: 2-5-2 Marunouchi, Chiyoda-Ku, Tokyo

Name: Mitsubishi Chemical Corporation